STANDARDIZATION AND EVALUATION OF THE *CATHARANTHUS ROSEUS* EXTRACT

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With the name of Allah, Who is utmost kind and merciful

To my family, Suny, Sabo and Sado

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LIST OF ABBREVIATIONS

Abbreviations	Description
AEC	Animal Ethics Committee
aFGF	acidic fibroblast growth factor
ACPE	Accreditation Council for Pharmacy Education
ANOVA	Analysis of Variance
AOT	Acute Oral Toxicity
ATCC	American Type Cell Culture Collection
BCNU	Bis Chloroethyl Nitrous Urea
bFGF	basic fibroblast growth factor
Bpm	Beats per minute
Bw	Body weight
CAM	Chick chorioallantoic membrane
CAMs	Complementary and Alternative Medicines
CCM	Chick Chorioallantoic Membrane
cdk	Cycline-dependent kinase
CDER	Center for Drug Evaluation and Research
CMC3	Chemistry Manufacturing Control Coordinating Committee
C roseus	*Catharanthus roseus*
CSO	Cage Side Observations
CTR	Catharanthine
CV	Coefficient of Variation
CVT	Cell Viability Test

CYP3A4	Cytochrome P450, family 3, subfamily A, Polypeptide 4
cyt. Arabin	Cytosine Arabinoside
DMSO	Dimethylsulphoxide
DNA	Deoxyribo Nucleic Acid
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic acid
EMA	European Medicines Agency
FBS	Foetal Bovine Serum
FDA	Federal Drug Administration
FDP	Fixed Dose Procedure
G	Gavages (feeding tube)
G1 Phase	Gap 1 phase
GC	Gibco, Canada
GCP	Good collection practice
GHS	Globaly Harmonized System for classification and labeling
GI	Gastro Intestinal
GIT	Gastro Intestinal Tract
GLP	Good Laboratory Practice
HCT-116	Colon carcinoma cells
HPLC	High Performance Liquid Chromatography
HT-29	Colon carcinoma cells
IACUC	Institutional Animal Care and Use Committee
IC_{50}	The half maximal inhibitory concentration
ICH	International Conference of Harmonization

IPPT	Institut Perubatan dan Pergigian Termaju (Advanced Medical and Dental Institute)
IPS	Institut pengajan siswazah (Institute of Postgraduate Studies
LD50	Lethal dose for 50% of population
LOD	Limit of Detection
LOQ	Limit of Quantification
m	Slope of the calibration curve
MAKNA	Majlis Kanser Nasional
MDR	Multi drug resistance
$MELD_{10}$	Mouse equivalent LD_{10}
M phase	Mitotic phase
MRP	Multi-drug Resistance-associated Proteins
MIAs	Monoterpenoid indole alkaloids
MTAs	Microtubules targeting agents
MTD	Maximum tolerated dose
MTT	Methyl thiazoldiphenyl tetrazolium
NCI	National Cancer Institute
NY	New York
N mustard	Nitrogen mustard
OD	Optical density
OCDE	Organisation de coopération et de développement économiques
OECD	Organization of economic cooperation and development
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline

Pgp	P-glycoprotein
PDA	Photo diode array
PMS	Phenazine methosulphate
RAM	Rat aorta model
R^2	Correlation coefficient
RO	Reverse osmoses
RPC	Reversed phase chromatography
RSD	RSD relative standard deviation
Rt	Retention time
SD	Starting dose
SD	Standard deviation
S phase	Synthetic phase
3T3	Normal cells (colon)
TSGs	Tumor suppressor genes
T-47D	Breast carcinoma cells
Tc	Test animal (control)
Tm	Test animal (male)
Tf	Test animal (female)
TSGs	Tumor suppressor genes
UDP	Up and down procedure
USFDA	United States Food and Drug Authority
USM	University Sains Malaysia
UWL	Unstirred water layer
VEGF	Vascular endothelial growth factors
VRLP	*Vinca rosea* leaves extract

VBL	Vinblastine
VCR	Vincristine
VDL	Vindoline
WHO	World Health Organization

LIST OF SYMBOLS

Symbol	Description
∞	Alpha (unclassified)
m	Slope of the calibration curve
I	Index for the day
S	Slope
σ_D	Standard Deviation
σ^2_D	Day to day variability
σ^2_e	Within day or intraday variability.
σ	Sigma (Standard deviation of the response that is y- intercept)
$10\ \sigma_D$	Relative standard deviation is 10 % exhibiting a signal to noise ratio 0.1
μ	Micro
μ	Mu
μ_z	Unknown parameter

PEMPIAWAIAN DAN PENILAIAN EKSTRAK *CATHARANTHUS ROSEUS*

ABSTRAK

Satu ekstrak piawai *Chantarantus roseus* telah disediakan dengan menggunakan kaedah pengektrakan air dan ethanol. Kandungan bahan aktif ekstrak telah ditentukan dengan kaedah HPLC. Jumlah alkaloid *Vinca* yang digunakan sebagai bahan antikanser vinblastina (VBL) dan vincristina (VCR) telah ditentukan. Kandungan VBL dan VCR ditentukan untuk menilai sumbangan alkaloid-alkoloid ini terhadap aktiviti anti kanser ekstrak yang dihasilkan. Pengesahan kaedah HPLC dilakukan berdasarkan prosedur yang terdapat dalam garispanduan *USFDA* dan *ICH*, dan didapati efisien dan boleh dihasilkan semula. Had minimum yang boleh dikesan untuk VCR adalah 0.25 μg/ml danVBL ialah 0.5 μg/ml. Kelinearan yang boleh diterima dipamerkan dengan pekali regresi (R^2), iaitu 0.9999 hingga 1. Alkaloid pertama yang dielut adalah vinkristina dengan masa retensinya (R_t) adalah 3.2.minit, diikuti dengan vindolina 3.56 minit, vinblastina 3.89 minit dan katarantina 4.14 minit. Analisis HPLC daripada ekstrak *Catharanthus roseus* menunjukkan bahawa kandungan VBL adalah sama dalam semua kelompok ekstrak, namun demkian kepelbagaian dalam kandungan VCR turut diperhatikan. Kandungan ekstrak *Catharanthus roseus* yang terkira adalah rendah. Keputusan ekstrak etanol E_1, E_3, E_4, E_6 dijulat sebagai 11.9μg/10mg, 9.0μg/10mg, 12.3μg/10mg dan 14.4μg/10mg bagi vinblastina dan kandungan ekstrak air W_1, W_3, W_4, W_6 dijulat sebagai 8.2μg/10mg,11.0μg/10mg,10.3μg/10mg dan 7.5μg/10mg bagi vinblastina. Keputusan ekstrak E_1, E_3, E_4, E_6, W_1, W_3, W_4, W_6 dijulat sebagai 19.9μg, 13.2μg, 8.1μg, 26.3μg, 8.2μg, 13.1μg, 11.1μg, and 11.6 μg/10mg bagi vincristina. Meskipun fakta bahawa kandungan alkaloid wujud dalam ekstrak *Catharanthus roseus*, namun ia adalah rendah apabila terdedah terhadap lini sel kanser yang berbeza, ekstrak WI, WII, W_6 dan E_6 mempamerkan aktiviti antikarsinogen. Kajian lini sel menunjukkan bahawa semua ekstrak yang terpilih untuk kajian ini adalah agen sitotoksik yang baik. Ekstrak etanol (E_6) menunjukkan aktiviti kesitotoksikan yang boleh diterima terhadap sel kanser payu dara T47D (IC_{50} 12μg/ml). Ekstrak air (W_6) menunjukkan aktiviti yang boleh diterima terhadap kedua-dua lini sel kanser kolon

HCT116 (IC_{50} 10μg) dan HT29 (IC_{50} 1.98μg). Ekstrak WI (etanol/air) juga aktif terhadap kedua-dua lini sel kanser kolon HCT116 (IC_{50} 12.28μg) dan HT29 (IC_{50} 1.65μg). Ketoksikan oral akut adalah ketoksikan yang terhasil dalam tempoh tidak melebihi 24 jam, selepas sesuatu drug yang diberikan. Semasa ujian, pemerhatian tepi-sangkar (cage side observations, CSO) dilakukan secara berterusan untuk menentukan ketidaknormalan dan kemorbidan. Pembolehubah berat badan dan pengambilan makanan diawasi dengan teliti. Tiada perubahan dalam pembolehubah ini ditemui. Perubahan dalam pembolehubah ini diketahui sebagai tanda ketoksikan pertama. Kajian Ketoksikan Oral Akut Dos Tunggal menunjukkan bahawa ekstrak air dan etanol didapati tidak toksik jika diberi secara oral. Kajian angiogenesis menunjukkan bahawa larutan ekstrak *Catharanthus roseus* boleh merencat pertumbuhan saluran darah. Aktiviti antiangiogenesis dalam ekstrak diuji menggunakan Model Aorta Tikus (Rat Aorta Model RAM). Diperoleh bahawa, ekstrak ini merupakan agen antiangiogenesis yang baik pada kepekatan 10 μg per ml. Oleh itu, daripada kajian *in vitro*, *in vivo* dan *ex vivo* dapat disimpulkan bahawa ekstrak *Catharanthus roseus* adalah sitotoksik bagi lini sel kanser, tidak toksik apabila diberikan secara oral kepada haiwan dan juga mempunyai aktiviti antiangiogenesis.

STANDARDIZATION AND EVALUATION OF THE EXTRACT OF *CATHARANTHUS ROSEUS*

ABSTRACT

A standardized *Catharanthus roseus* extract has been prepared by water and ethanol method. The widely used anticancer *Vinca* alkaloids, vinblastine (VBL) and vincristine (VCR) present in the extract were determined by using HPLC. Contents of VBL and VCR were determined to assess the contribution of these alkaloids towards the anticancer activity of the extract. The method was validated in accordance to the procedure mentioned in the Guidelines of *USFDA* and *ICH*. The method was found efficient and reproducible. The minimum detectable limit of VCR was 0.25 μg/ml and VBL was 0.50 μg/ml. An acceptable linearity was exhibited with regression coefficient (R^2) of 0.9999 to 1. The first eluted alkaloid was vincristine its retention time (R_t) was 3.2 minutes followed by vindoline 3.56 minutes, vinblastine 3.89 minutes and catharanthine 4.14 minute. The content of VBL for the ethanol extracts E_1, E_3, E_4 and E_6 were 11.9μg/100mg, 9.0μg/100mg, 12.3μg/100mg and 14.4μg/100mg of the dried extract. The contents of VBL determined in water extracts W_1, W_3, W_4, W_6 were 8.2μg/100mg, 11.0μg/100mg, 10.3μg/100mg and 7.5μg/100mg. Amount of VCR present in *Catharanthus roseus* extracts E_1, E_3, E_4, E_6, W_1, W_3, W_4, W_6 was determined as 19.9μg, 13.2μg, 8.1μg, 26.3μg, 8.2μg, 13.1μg, 11.1μg, and 11.6 μg/100mg of the dried extract. The study of the extract on cancer cells demonstrated good cytotoxic activity. Ethanol extract E_6 showed acceptable cytotoxicity against breast cancer cells T47D (IC_{50} 12μg/ml). Water extract W_6 showed pronounced anticancer activity against colon cancer cell lines HCT116 (IC_{50} 10 μg/ml) and HT29 (IC_{50} 1.98 μg). Ethanol/water extract WI was also active against both colon cancer cell lines HCT116 (IC_{50} 12.28 μg) and HT29 (IC_{50} 1.65 μg). In single dose acute oral toxicity test, variables (body weight and feed intake) were carefully checked and no significant changes in these variables were initiated. There was no difference in physical state, behavior and response of the control and the treated groups. This study indicated that water and ethanol extracts were less toxic and can be administered orally. Antiangiogenesis study in Rat Aorta Model (RAM) revealed that the 10μg/ml solution of the alcohol

water extract WI and Ethanol extract E_6 of *Catharanthus roseus* inhibited the growth of blood vessels. The extracts appeared to be good antiangiogenic agent. The current study indicated that the *Catharanthus roseus* extract is less toxic when administered orally, exhibited cytotoxicity on cell lines and demonstrated good anti-angiogenesis activity.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE SURVEY

1.1 CANCER

Cancer is produced from a normal cell when it becomes abnormal. Loss of growth control in a single cell causes trillions of cells to arise from a single cell (Bissell, 1981). The body has an unknown mechanism to eliminate cells losing growth control, but sometimes due to a variety of chromosomal changes, the cell cycle is driven crazy into nonstop division. The transcription factors such as E2F and c-myc are responsible for the cell cycle or cell growth, and involve in forcing cells into apoptosis or cell death. Thus, cell birth and cell death are initiated by the same pathway (Evan and Littlewood, 1998; King and Cidlowski, 1998).

Cancer results from the genetic and epigenetic abnormalities in susceptible cells (Ponder, 2001; Houghton *et al*., 2007). In general, somatic mutations are involved, but it can also be inherited. Linkages are found through linkage-analysis of families with inherited predisposition to cancer (Wooster *et al.*, 1995). Dietary and environmental factors also contribute in cancer formation (Doll and Peto, 1981; Houghton *et al*., 2007). Cancer is proven to be a potentially fatal disorder that involves proliferation, transformation and deregulations of apoptosis. Cancer genes initiate extensive changes in cellular structure and cellular functions. These changes increase sensitivity to growth signals and insensitivity to growth-inhibitory signals,

decrease apoptosis and increase potential to replicate, generate angiogenesis and metastasis (Hanahan and Weinberg, 2000; Ichikawa *et al.*, 2007).

1.1.1 Oncogenes and tumor suppressor genes

Three types of genes are known to take part in development of cancer. The first type is oncogenes, is mutated or over expressed from normal proto-oncogenes. These are positive regulators of cell growth for example, n-*myc* in neuroblastoma, c-*erb*-B2 in breast cancer and k-*ras* in pancreas cancer. The second type is known as tumor suppressor genes (TSGs). These are negative regulators of cell growth (Knudson, 1971). The third type is DNA repair genes (Cleaver, 1994). The tumor originates from the activation of oncogenes and the inactivation of tumor-suppressor genes.

Over hundred oncogenes have been identified and can be defined as gene-encoding products. The increased activity of oncogenes leads to increased proliferation. The deregulation of cancer genes results in a wide range of changes in cellular function and structure, all are contributing in various ways to malignancy (Hanahan and Weinberg, 2000). Some oncogenes, such as *ras*, *myc* and *erb* are identified as the human homologs of the viral transforming genes. Cellular genes become activated-oncogenes when these are incorporated into the viral genome (Harkin and Johnston, 2005).

1.1.2 Molecular natural cell cycle clock

A molecular natural clock instructs the cell when to replicate its DNA and when to divide. Some proteins regulate the timings of events occur in the cell cycle. The loss of control on timing leads towards cancer (Houghton *et al.,* 2007; Ichikawa *et al.,* 2007). A protein known as *cycline* is the oscillator that runs the molecular clock. The cellular concentration of *cyclin* increases till the time of cell division and then suddenly decreases (Evan *et al.*, 1983). One specific enzyme known as *cyclin*-dependent kinase (cdk) also takes active part in the clock mechanism. Then another component protease (in the form of a proteasome) reset the clock (Glotzer *et al.,* 1991; King *et al.,* 1996). The cell cycle time for human tumors is around two days to several weeks (Wilson *et al.,* 1988; Basse *et al.,* 2002).

1.1.3 A brief history of treatment of cancer

In early days, the bacterial toxins were used in cancer treatment. It was based on host–tumor interactions, which opens the way to anti-angiogenesis and immune approaches. Modern research is developing a combination of complex strategies for growth and death control systems within the tumor cell. However, the development of low molecular-weight anticancer drugs and new strategies are likely to be continued. Currently the treatment for cancer ailment involves a combination of surgery, radiotherapy and chemotherapy. Natural products from plant origin play a very significant role in cancer therapy. One of the flowering plants, *Catharanthus roseus* is fundamentally cytotoxic and inhibits proliferation by exhibiting a very unique strategy, known as cell cycle specificity (Johnson *et al.,* 1963). A brief history of clinical cytotoxic drugs is summarized in the following Table 1.1

Table 1.1 A brief history of clinical cytotoxic drugs

No	Drug	Approximate Year of introduction
1	N Mustard	1948
2	Methotrexate	1953
3	Chlorambucil	1956
4	Thioguanine	1958
5	Cyclophosphamide	1959
6	Vinblastine	1960
7	5-Fluorouracil	1961
8	Vincristine	1962
9	Melphalan	1964
10	Daunorubicin	1965
11	Cytarabine.	1970
12	Bleomycin	1974
13	Doxorubicin	1976
14	Mitomycin	1977
15	Dacarbazine	1978
16	BCNU	1980
17	Cisplatin	1978
18	Etoposide	1979
19	Teniposide	1980
20	Amsacrine	1981
21	Carboplatin	1984
22	Epirubicin	1987
23	Mitoxantrone	1989
24	Paclitaxel	1992
	WHO recognized this list of 24 essential drugs	1994
25	Gemcitabine	1995
26	Docetaxel	1996
27	Topotecan	1997
28	Irinotecan	1998

No	Drug	Approximate Year of introduction
	WHO updated the list into three categories	1999
	Category 1 Essential drugs	1999
1	Bleomycin	
2	Chlorambucil	
3	Cisplatin	
4	Cyclophosphamide	
5	Cytarabine	
6	Dactinomycine	
7	Daunorubicin	
8	Doxorubicin	
9	Etoposide	
10	Fluorouracil	
11	Mercaptopurine	
12	Methotrexate	
13	Prednisolone	
14	Procarbazine	
15	Tamoxifen	
16	Vinblastine	
17	Vincristine	
	Category 2 12 drugs (See Martindale, 2007)	1999
	Category 3 Non essential 13 drugs (See Martindale, 2007)	1999

1.2 DRUG DISCOVERY OF NATURAL FLORA

Nature contributes a very significant role in medicinal therapy. It is likely to continue to be a source of new drugs in this modern era (Shah and Kaye, 2003). Natural compounds are complexes of well kept secrets of nature. Researchers are disclosing these wonders by using different techniques through available knowledge in the field of drug discovery to select out the compound of their interest. Current high throughput instrumental development had made drug discovery and development process more efficient (Drager, 2002; Houghton, 2002; McCally, 2002; Molyneux *et al.*, 2002; Stockigt *et al.*, 2002; Chen *et al.*, 2004; Balunas; King-Horn, 2006).

1.2.1 Lead structures of the synthetic compounds

Lead structures of most synthetic chemicals are based on structure of natural products (Mann, 2002). The supremacy of natural products over synthetic compounds is due to their colossal structural and chemical diversity. ―About 40% of the chemical scaffolds found in natural products are not available in today‘s medicinal chemistry" (Muller-Kuhrt, 2003). More than 50% of the drugs in clinical use are obtained from plant origin (Mann, 2002). According to the world health organization (WHO), up to 80% of people living in developing countries are following Traditional Medicine for their healthcare. People believe that majority of the natural products used are pharmacologically safer, more affordable and have in-built advantage that they hit multiple targets. (Kong and Liu, 2006)

1.2.2 "Natural Inhibitor of Carcinogenesis"

More than 10 countries have collaborated with the United States to conduct research under the project of "Natural Inhibitor of Carcinogenesis" and listed more than 250 compounds out of 5000 plant samples as potential cancer chemoprevention. The European Parliament passed a new legislation for European Union member nations to facilitate traditional medicine makers in terms of determination of efficacy. Canada opened a new Natural Health Products Directorate programme in January 2004. Pakistan, India, and Brazil are also developing botanical-drug research and testing centers (Kong and Liu, 2006). More than 2000 species grown in Malaysia are reported to have medicinal value (Jaganath and Ng, 2000). The climate of Malaysia is also suitable for the growth of *Catharanthus roseus* (*C. roseus*).

1.3 *CATHARANTHUS ROSEUS* (*C. ROSEUS*)

Catharanthus roseus is an ever blooming sub-tropical shrub. It was originally indigenous to Madagascar. It is now widely grown and used as indigenous medicine all over the world (Yarnell and Philhower, 2005).

1.3.1 Nomenclature of *Catharanthus roseus*

Its official name is *Catharanthus roseus* Linn G. Don, from family Apocynaceae (Leveque and Jehl, 2007; Magnotta *et al.*, 2007). Its synonym is *Vinca rosea* Linn, other included names are Lochnera rosea Reichb (Daniel, 2000), Periwinkle, Baramasi and Rattan-jot (Chopra *et al.*, 1956 and1986). In Pakistan this plant is locally named as *Sadaa bahaar* (ever blooming). In Malaysia it is locally

known as *Kemuning cina* (Siddiqui, 2010). It produces potent anticancer *Vinca* alkaloids (Cutts *et al.*, 1960: Johnson, *et al*., 1963).

Figure 1.1 *Catharanthus roseus*

1.3.2 Ethno-medical uses of *Catharanthus roseus.*

Most of the useful drugs derived from plants have been discovered by follow-up of the ethno medical uses (Farnsworth *et al.*, 1985; Dutcher *et al*., 2000). In 1910, *Catharanthus roseus* was reported to be useful in Brazil as infusion of the leaves for mouth washes and toothaches, for the cleansing and healing of chronic wounds and also used in hemorrhage and scurvy (Synold, 2005; Johnson *et al*., 1960). In British West Indies, it has been used to treat diabetic ulcers (Johnson *et al*., 1960) In the Philippines; it has been used orally in the treatment of hyperglycemia and hypertension (Garcia, 1953).

In the forests of Madagascar, local people have been using decoction of the roots of *Catharanthus roseus* for the treatment of parasitic worms (Norscia and Borgognini-Tarli, 2006). Water extract of *Catharanthus* roseus was used for bleeding arrest, diabetes and fever or rheumatism (Ross, 2003). The leaves of the plant were chewed to suppress the sensations of hunger and fatigue (Ross, 2003; van der Heijden *et al.,* 2004; Ferreres *et al.,* 2008). The herbal and other preparations of *C. roseus* have been used for cancer and hypertensive treatment since ancient times (Hardman and Limbird, 2001).

1.4 *VINCA* ALKALOIDS AND OTHER COMPOUNDS PRESENT IN *CATHARANTHUS* ROSEUS (*C. ROSEUS*)

Antihypertensive/antiarrhythmic alkaloid ajmalicine and tranquillizer alkaloid serpentine and anticancer alkaloids vincristine and vinblastine isolated from *Catharanthus roseus* (C. *roseus*) were introduced as *Vinca* alkaloids (Hardman and Limbird, 2001). Developments in cancer treatment started in the 20th century (Baguley, 2002).

More than hundred indole *Vinca* alkaloids are reported from *Catharanthus roseus* (Heijden *et al.,* 2004; Hisiger and Jolicoeur, 2007). Twenty Indole-indoline dimeric alkaloids are more important. They are listed in Table 1.2. Only serpentine as tranquillizer (Iwase *et al.,* 2005), ajmalicine as anti-arrhythmic (Srivastava *et al.,* 2006) and vinblastine, vincristine and 3, 4- anhydrovinblastine as anti-cancer were marketed (Heijden, *et al.* 2004). Vindoline and catharanthine are the precursors in the biosynthetic pathways of dimeric indole alkaloids vinblastine and vincristine (Noble, 1990; Jolicoeur, 2007). *Catharanthus roseus* plant gains more interest due to its anticancer alkaloids (Sousa *et al*., 2008). A list of anticancer alkaloids is mentioned bellow in Table 1.3.

Table1.2 Important alkaloids reported from *Catharanthus roseus*

Alkaloids	Old **name**	**Source**	**Empirical Formula**
Vinblastine	Vincaleukoblastine	Whole plant	$C_{46}H_{58}N_4O_9$
Vincristine	Leurocristine and Vincaleurocristine	Arial parts	$C_{46}H_{56}N_4O_{10}$
Vindesine	Vindesine	Arial parts	$C_{43}H_{57}N_5O_{11}S$
Leurosine	Leurosine	Arial parts	$C_{46}H_{58}N_4O_9$
Ajmalicine	Raubasine	Flowers	$C_{21}H_{24}N_2O_2$
Lochnerine	Lochnerine	Flowers	$C_{20}H_{24}N_2O_2$
Lochnericine	Lochnericine	Flowers	$C_{21}N_{24}N_2O_2$
Catharanthine	Catharanthine	Flowers	$C_{21}H_{24}N_2O_2$
Serpentine	Serpentine	Flowers	$C_{21}H_{22}N_2O_3$
Vindoline	Vindoline	Arial parts	$C_{25}H_{32}N_2O_6$
Vindolinine	Vindolinine	Arial parts	$C_{21}H_{24\text{-}6}N_2O_2$
Reserpine	Reserpine	Arial parts	$C_{23}H_{40}N_2O_9$

(Johnson, *et al.,* 1960; Daniel, 2000; British Pharmacopoeia, 2000; Dutta *et al.,* 2005)

Table 1.3 Frequently used salts of anticancer alkaloids from *Catharanthus roseus*

Salts of anticancer alkaloids	**Brand name**	**Molecular weight of salt**	**Empirical formula**
Vinblastine sulfate	Exal,Velban Velbe Velsar	909.1 (sulfate)	$C_{46}\ H_{58}\ N_4\ O_9,H_2SO_4$
Vincristine sulfate	Oncovine, Vincasar, Kyocristine,Vincosid, Vincrex	923 (sulfate)	$C_{46}H_{56}N_4O_{10},H_2SO_4$
Vindesine sulfate	Eldistine, Fildesine	852 (sulfate)	$C_{43}H_{57}N_5O_{11}S$
Vinorelbine ditartrate	Navelbine	1079 (di tartrate)	$C_{45}H_{54}N_4O_8,2C_4H_6O_6$

(Johnson, *et al.,* 1960; Daniel, 2000; British Pharmacopoeia, 2000; Dutta *et al.,* 2005)

The anticancer activity of the *C. roseus* is mainly dependent on alkaloids such as vincristine, vinblastine, vindesine and vinorelbine, but the presence of phenols (Piovan and Filippini, 2007; Mustafa and Verpoorte, 2007; Ferreres *et al.,* 2008) and antioxidants (Misra and Gupta, 2006; Abduljaleel and Gopi, 2008) also contribute highly towards anticancer activity. The known compounds other than alkaloids and their mechanism of action are discussed in Section 1.10.2.

1.4.1 Discovery of anticancer alkaloids from *C. roseus*

Vinca alkaloids were isolated form *C. roseus* leaves in the late 1950s independently by two groups; Professor Noble working at the Western University of Ontario, Canada, and the other researchers at the Eli Lilly research laboratories in Indianapolis, USA (Noble, 1958 and 1990; van der Heijden *et al.*, 2004). *Vinca* alkaloids were scientifically studied initially as oral hypoglycemic agents (Johnson *et al.*, 1963).

Robert Noble was working in Canada on some hypoglycemic oral compound found in *Catharanthus roseus,* (*C. roseus*) not grown in Canada (Tailor and Farnsworth, 1975). *C. roseus* extract solution when injected into mice caused immune suppression (Noble *et al.*, 1958; Duffin, 2002). It indicated the extract can induce pronounced activity without any toxic effect (Johnson *et al.*, 1963; Yarnell and Philhower, 2005).

1.4.2 Earliest chemotherapeutic agents

Catharanthus roseus is the only source for vinblastine and vincristine. *Catharanthus roseus* produced around 130 indole alkaloids, but a full picture of its biosynthetic pathway has yet to be elucidated (Zeffrin, 1984; El-Sayed and Verpoorte, 2007). Before 1961, vinblastine was the only anticancer drug commercially available for cancer treatment from *C. roseus.* In March 1961, Eli Lily announced the commercial availability of a new anticancer compound called leurocristine, which was the former name of vincristine (Johnson *et al*., 1960). Vinblastine and vincristine are among the earliest agents developed for cancer therapy. They are approved for cancer treatment for more than 30 years and remain widely used in anticancer therapy.

1.4.3 Cancers treated by *Vinca* alkaloids

Vinca alkaloids and their derivatives act as mitotic inhibitors (Rowinsky and Donhower, 1996). Vinblastine and vincristine have been used as chemotherapeutic agents in the treatment of a wide range of tumors, particularly childhood tumors, leukemia and lymphomas (Riyaz and Stanley 2003; Synold, 2005; Levêque and Jehl, 2007), Hodgkin‘s disease (Svoboda, *et al.*, 1959; Levêque and Jehl, 2007) and testicular teratoma (Mann, 2002; Synold, 2005). It is also effective in treating breast carcinoma, nephroblastoma, brain tumors, lung cancer, leiomyosarcoma, cervix cancer, alimentary tract tumor (Pui and Evans, 1998) and Kaposi‘s sarcoma (Kaplan *et.al.*, 1986; Gill *et al.*, 1991; Tulpule and Matheny, 1998). Different cancers treated by *Vinca* alkaloids are listed in Table 1.4

Table 1.4 Cancers treated by *Vinca* alkaloids.

Alkaloid	Uses
Vinblastine Mitotic inhibitor	Leukemia, Hodgkin's lymphoma and germ cell cancer, Kaposi's sarcoma, bladder, breast and lung cancer.
Vincristine Topoisomerae inhibitors	Ovarian cancer, leukemia, Hodgkin's lymphoma, Wilm's tumor, multiple myeloma, breast and lung cancer.
Vinorelbine (Navalbine)	Breast, non-small cell lung cancer, gastrointestinal cancer
Vinoflunine Phase-II clinical trials	Phase-II clinical trials bladder, non-small cell lung carcinoma and breast cancers.

(Mac Lenna and Cusack, 1985; Boccardo *et al.*, 1989; Daniel, 2000; British Pharmacopoeia, 2002; Extra Pharmacopoeia, 2002; Ichikawa *et al.*, 2007)

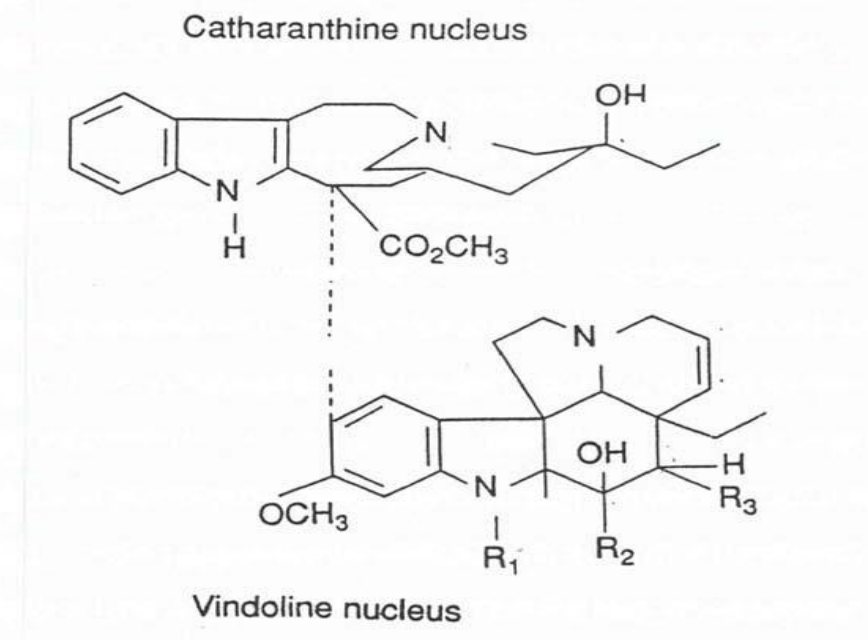
1.4.4 Sulfate and ditartrate salts of *Vinca* alkaloids

In treatment of the cancer, either of the *Vinca* alkaloids is prescribed in combination with the other cytotoxic drugs. A collection of alkaloids from *Catharanthus roseus* (*C. roseus*) is presented in Table1.2. *C. roseus* produced various monoterpenoid indole alkaloids (MIAs) (Ichikawa *et al.*, 2007). They are utilized in salt form as listed in Table1.3. The sulfate salts of vinblastine, vincristine and vindesine are administered via intravenous route. Vinorelbine ditartrate salt (Novelbine) is administered orally (Degardin *et al.*, 1994; Extra Pharmacopoeia, 2002). A semi synthetic fluorinated *Vinca* alkaloid is produced through change in the catharanthine moiety of vinblastine (Kruczynski and Hill, 2001; Jacquesy, 2006).

1.5 STRUCTURE OF *VINCA* ALKALOIDS

A group of researchers at Eli Lilly discovered the structures and revealed the clinical activity of vincristine and vinblastine (Edwards, 1994). *Vinca* alkaloids are identical in structure with difference in the group attached to the nitrogen at position 1 at which vincristine possesses a labile N- formyl group and vinblastine has a stable methyl group Figure 1.2. D ring is a small portion of *Vinca* molecule which is extremely sensitive in term of overall biological properties and tubulin interactions (Jacques, 2001).

Fig 1.2 Chemical structure of *Vinca* alkaloids



Alkaloids	**R1**	**R2**	**R3**
Vinblastine	-CH3	-CO2CH3	-OCOCH3
Vincristine	-CHO	-CO2CH3	-OCOCH3
Vindesine	-CH3	-CONH2	-OH
Vinorelbine	-CH3	-CO2CH3	-OCOCH3

1.5.1 Structural difference and clinical activity

There are minor structural differences present between anticancer *Vinca* alkaloids and they behave in the same way with the drug-tubulin interaction but their toxicity and spectrum of clinical activity is significantly different from each other. (Himes *et al.*, 1976; Owellen *et al.*, 1977)

1.5.2 Derivatives of *Vinca* alkaloids

In combination with other anticancer therapies, vinblastine and vincristine are given for a curative purpose (Levêque & Jehl, 2007). From vinblastine (Oncovin) and vincristine (Velbe), a large number of derivatives have been formed. They are modified in the vindoline moiety (mido derivative). Vindesine (deacetylvinblastine amide) (Barnett *et al.,* 1978) was registered in Europe in 1980. Vindesine is not approved in the United States, and in France. It is used in the treatment of aggressive forms of non-Hodgkin lymphomas in combination with other anticancer drugs.

In 1980s a semi synthetic derivative is introduced as vinorelbine nor-5′-anhydrovinblastine (Navelbine). It is invented by the pharmacist Pierre Potier and his team in France, produced by changing the catharanthine moiety of vinblastine. Vinorelbine is given in palliative treatment of advanced nonsmall-cell lung cancer, refractory lymphoid malignancies and advanced breast cancer. Vinorelbine gives greater therapeutic activity when given as adjuvant (or postoperative) treatment associated with cisplatin in patients with resected non-small-cell lung cancer (Winton *et al.,* 2005; Douillard *et al.,* 2006).

Vinflunine is a fluorinated derivative of vinorelbine. It is in the process of clinical development (Kruczynski & Hill, 2001; Bennouma *et al.,* 2005). The substitution of fluorine in the structure of natural products has been proven beneficial. The fluorination increases the lipophilic profile of a molecule, which can be more effective (Thomas, 2006). Novel vinflunine (Javlor) is under clinical trial (Kruczynski and Hill, 2001; Jacquesy, 2006) and advanced to the phase III clinical trials (Shnyder, 2004). Vinflunine hold a potent antiangiogenic effect (Holwell *et al.*, 2001).

1.6 MODE OF ACTION OF *VINCA* ALKALOIDS

The mechanism of antineoplastic activity is worked during cell mitosis (George *et al.*, 1965). *Vinca* alkaloids are mitotic inhibitors (Himes, 1991; da Rocha *et al.*, 2001; Honore *et al*., 2003). These agents target microtubules. They are the structural backbone of both normal and abnormal cells. Microtubules are polymers of long tube shaped dynamic structures. They are constantly growing and shortening (Mitchison and Kirachner, 1984).

Microtubules contribute essential roles in construction and function of the mitotic spindle. They actively take part in many cellular events, including cellular organization, cell division, intracellular transport, intracellular transfer of signals, neurotransmission, and the transmission of signals from the cell-surface-receptors to the nucleus (Dutcher and Novik, 2000).

1.6.1 Target of *Vinca* alkaloids for anticancer activity

Tubulin is one of the essential proteins for chromosomal segregation. The tubulin- microtubule system is an important target for anticancer therapy (Jordan and Hadfield, 1998; Houghton, 2002). Hydrophobic *Vinca* alkaloids bind to tubulin in a reversible manner (Zhou and Rahmani, 1992). The *Vinca* alkaloids inhibit the microtubule polymerization at high non-physiologically relevant concentrations (Jordan *et al*., 1991; Dhamodharan, *et al.,* 1995). *Vinca* alkaloids act by binding to the micro-tubular proteins of the spindle and arresting mitosis at the metaphase/anaphase transitions leading to apoptosis (Nagan, 2000; Kruczynski *et al.* 2002).

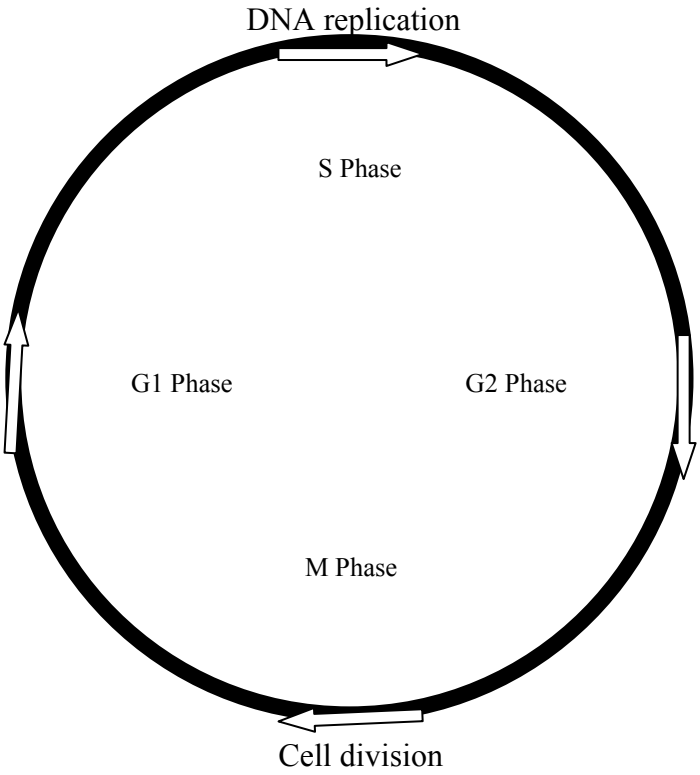
The mechanism of the mitotic blockage is not well known (Okouneva *et al,* 2005; Weaver and Cleveland, 2005), the *Vinca* alkaloids interfere with the function of microtubules in axons, which mediates the neuronal vesicle transport (Dumontet *et al.*, 1999; Bacher *et al.*, 2001). The research is performed on an array of chemically diverse antimitotic and microtubule targeting agents. They depolymerize microtubules of newly developed vascular system and stop the blood supply to tumor (Okuneva *et al.,* 2003; Honore *et al.,* 2003; Jordan and Wilson, 2004). The function of *Vinca* alkaloids as angiogenesis agent is discussed in chapter 6.

1.6.2 *Vinca* alkaloids as mitotic inhibitors during cell cycle

There are two prominent phases occur in mammalian cell division. One is known as synthetic or S phase and the other is mitosis or M phase. There are two gaps G1 and G2 occur between S phase and M phase. *Vinca* alkaloids function as mitotic inhibitors between G2 and M phase of the cell cycle. (Musunru and Hinds,

1997) The transitions from *G*1-phase to *S*-phase, and from *G*2-phase to *M*-phase, are controlled by a system of cyclin-dependent kinases (cdk), cyclins and phosphatases (Houghton *et al.,* 2007; Ichikawa *et al.,* 2007). The cell cycle clock is demonstrated in Figure 3.

Figure 1.3 The cell cycle clock



S = synthetic

G = gap

M = mitosis

The known mechanism of drug action is to arrest cells at the metaphase by interfering with the assembly or disassembly of ∝- and *β*- tubulin into microtubules and inhibits tubulin polymerization (Rowinsky and Donhower, 1996). These drugs target β-tubulin subunit of ∝-/β- tubulin heterodimers, inhibiting the addition of heterodimers onto growing microtubules, hence, preventing polymerization of microtubules (Musunru and Hinds, 1997; Jordan and Wilson, 1998).

1.6.3 Activity of *Vinca* alkaloids in high and low concentration

At high concentrations, the *Vinca* alkaloids cause complete microtubule de-polymerization. At low concentrations de-polymerization does not occur but there is sufficient alteration in the dynamics of tubulin loss or addition at the ends of mitotic spindle. It prevents the spindle from carrying out its function of attaching to and segregating the chromosomes, and cause cell arrest during mitosis (Jordan *et al*., 1992; Jordan, 2002; Jordan and Wilson, 2004). Prolonged arrest finally leads to cell death, either in mitosis or after an ultimate escape from mitotic arrest (Jordan and Wilson, 1998).

Lower concentrations of microtubule-targeted drugs can suppress dynamics of microtubule without changing mass of microtubule (Okouneva *et al.,* 2003). Tubulin binding and disruption of the cell membranes simultaneously occur due to interference with the lipid bi-layer at the same concentration ((Rowinsky and Donhower, 1996).

1.6.4 Special *Vinca*-specific high-affinity and low-affinity sites

Each heterodimer of tubulin possesses special *Vinca*-specific high-affinity and low-affinity sites. These binding sites are different from the interacting sites of other drugs such as taxanes (Rao *et al.*, 1992; Rao and Krauss *et al.*, 1994). Binding to the high affinity sites decreases the rate of dissociation and association of tubulin dimers of the microtubules (Jordan *et al.* 1986). Binding to the low-affinity sites appears to be responsible for disruption of the microtubule configuration, leading to disintegration (Jordan *et al.*, 1986).

Despite the above mentioned promising anticancer activity, these alkaloids have many problems during their uses. The drug resistance, toxicity, and low specificity are major difficulties in the treatment of cancer (de Mesquita *et al*., 2009). A brief account on these problems is discussed as follows.

1.7 CELL RESISTANCE AGAINST *VINCA* ALKALOIDS

The efficacy or activity of *Vinca* alkaloids decreases when cancer cells develop resistance against anticancer drugs (Dumontet and Sikic, 1999). When cancer cells develop clinical resistance to one drug, the simultaneous resistance to several other structurally and mechanistically unrelated drugs is also induced. Clinical resistance reduces the concentration of the drug in the target cell, which in consequence reduces activity and decreases the clinical effectiveness of drug (Husain and Wozniak, 1993; Hunter, 1997; ACPE, 2002).

1.7.1 Classical multiple drug resistance (MDR) of cancer cells to natural hydrophobic drug

Vinca alkaloids are comparatively more hydrophobic in nature. Resistance development of cancer cells to natural hydrophobic drugs are known as classical multi drug resistance. Study of cancer cells in culture with vinblastine, paclitaxel or doxorubicin frequently results in multi drug resistance due to expression of ATP-dependent efflux pumps with broad drug specificity. Family of ATP-binding cassette (ABC) transporters is responsible for efflux pumps. It is further divided into seven subfamilies (ABCA-ABCG). The ABC transporter P-glycoprotien (P-gp) is the product of the ABCB1 or *mdr1*-gene (Tsuruo *et al.*, 1972; Kolars et *al.*, 1992; Gottesman *et al.*, 2002).

There are different mechanisms of resistance including P-glycoprotien efflux pump and multi drug resistance protein (MRP). These proteins are transmembrane transporters which are also responsible for the rapid efflux of intracellular chemotherapeutical agents. Over expression of these proteins indicate the less concentration of intracellular *Vinca* alkaloids and reduction in cytotoxicity. In brief, the development of multiple drug resistance (MDR) is common for *Vinca* alkaloids due to the following reasons:

1. Over expression of transmembrane efflux pump system known as P-glycoprotein (P-gp) (Cole and Deeley, 1998; Bardelmeijer *et al.,* 2000; Bacher and Nickel, 2001).

2. The action of multi-drug resistance-associated proteins (MRP) (Fardel *et al.*, 1996; Cole and Deeley, 1998; Bardelmeijer *et al.,* 2000; Bacher and Nickel, 2001).